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Research Article

New *Helminthosporium* (Massarinaceae, Dothideomycetes) and *Nigrospora* (Incertae sedis, Sordariomycetes) species associated with walnut (*Juglans regia* L.) in China

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Abstract

Six collections of ascomycetes were obtained from samples collected from dead branches and leaves of *Juglans regia* in Guizhou and Yunnan provinces, China. By incorporating multigene phylogenetic analysis (ITS, LSU, *rpb2*, SSU, *tef1-α*, *tub2*) supplemented by morphological data, we establish two novel species, namely *Helminthosporium guizhouense* and *Nigrospora yunnanensis*. In morphology, *H. guizhouense* can be distinguished from *H. caespitosum* by its narrower conidia (13–16 µm vs. 27.3–35.5 µm), and *N. yunnanensis* is characterized by black, globose conidia (16.2 × 14.4 µm). The phylogenetic results further substantiated them as novel taxa. The present study contributes to our comprehension of the range of fungi found in *Juglans regia*, thereby expanding our knowledge of the diversity of fungi within this host.

Key words: Ascomycota, morphology, new taxa, phylogeny, taxonomy

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Introduction

Dothideomycetes and Sordariomycetes comprise plant pathogens, endophytes, and saprobes, and they can be identified by their distinct fruiting bodies (Marek et al. 2009; Wijayawardene et al. 2016; Hongsanan et al. 2020; Healy et al. 2022). They are widespread inhabitants of plant tissues including walnut (*Juglans regia* L.). Walnuts are a nutritious and health-beneficial drupaceous nut, globally recognized for their valuable properties (Caglarirmak 2003).

Helminthosporium (Massarinaceae, Pleosporales, Dothideomycetes) is a group of asexual Ascomycota proposed by Link (1809) with the type species *H. velutinum*. Most *Helminthosporium* species are saprobes that primarily inhabit various natural substrates, such as plant tissues, wood, bark, dung, and insects, and are also human pathogens (Luttrell 1964, Alcorn 1988; Konta et al. 2023; Hyde et al. 2023, 2024). About 781 taxa have been placed in *Helminthosporium* (http://www.indexfungorum.org, June.2024), but most *Helminthosporium*

species differ from the generic type in the development of conidia and conidiophores and therefore are excluded from *Helminthosporium*. Furthermore, very few taxa have molecular data (Hyde et al. 2023). Very few instances of sexual morphs of *Helminthosporium* have been recorded, and the validity of most of these records is questionable as they have not been confirmed by sequence data (Voglmayr and Jaklitsch 2017).

Nigrospora (Apiosporaceae, Xylariales, and Sordariomycetes) was proposed by Zimmerman (1902) with the type species *N. panici* (Hyde et al. 2024). Initially, the characterization of *Nigrospora* species relied on morphological features, particularly large dark conidiospores. However, it was discovered that certain key morphological characteristics, such as the size of spores, were similar among species that are actually not closely phylogenetically related (Hao et al. 2020). To accurately identify different species, it is important to use a comprehensive approach that combines both morphological characteristics and phylogenetic analysis (Jayawardena et al. 2020, Maharachchikumbura et al. 2021). Wang et al. (2017) revised the classification of *Nigrospora*, increasing the known species from 15 to 27 by incorporating morphological and molecular data. Chethana et al. (2023) and Hyde et al. (2024) added records of further species. Wang et al. (2017) confirmed the placement of the genus in Apiosporaceae (Xylariales) based on multi-locus molecular phylogeny, including the internal transcribed spacer (ITS), translation elongation factor 1-alpha (*tef1-α*), and b-tubulin (*tub2*) gene regions. This was confirmed by Samarakoon et al. (2021).

Southwest China is a biodiverse region. In this study, six isolates were collected from walnut leaves and dead tissues from Qianxi County, Guizhou Province, and Lincang City, Yunnan Province. This study aimed to determine the taxonomic status of the pathogenic species of walnut in Guizhou and Yunnan provinces through an analysis of both morphological and molecular characteristics. After conducting a multi-locus phylogenetic analysis and morphological examination, two new species, *Helminthosporium guizhouense*, and *Nigrospora yunnanensis* are identified and introduced.

Materials and methods

Sample collection, fungal strain isolation, and morphology

Samples exhibiting signs of disease were collected from walnuts in Qianxi County, Guizhou Province, and Lincang City, Yunnan Province, from 2023 to 2024. To establish uncontaminated cultures, disinfection processes were implemented on the sample surfaces (Zhang et al. 2020). Conidia were identified on the surface under a dissecting microscope. These conidia were aseptically extracted from the leaves using a sterilized needle and relocated to a sterile, water-filled drip board. The spores were then dispersed in sterile water, and a small quantity of the resulting spore suspension was absorbed and uniformly dispersed onto a potato dextrose agar (PDA) incorporated with streptomycin. After a 12-hour incubation period at 25 °C, individual germinated spores were selected and transferred to fresh PDA. Additionally, we prepared Malt Extract Agar (MEA) and Oatmeal Agar (OA) media for fungal growth. The cultures were subsequently maintained at room temperature (28 °C) for a duration of 10 days.

VHX-7000 (Keyence, Osaka, Japan), Fully-Integrated Head VHX-7100 (Keyence, Osaka, Japan), and High-Performance Camera VHX-7020 (Keyence, Osaka, Japan) dissecting microscopes were used as vehicles for observing the fungal colonies and fruiting bodies. The morphological characteristics of the fungi were studied and documented using a compound light microscope (Zeiss Scope 5) equipped with an attached camera (AxioCam 208 color). Morphological measurements of the new species' features were taken using the ZEN 3.0 (blue edition) (Jena, Germany) software. All newly identified taxa have been registered in the Mycobank database (https://www.mycobank.org), accessed on 28 June 2024. For long-term conservation and research purposes, dried holotype specimens were preserved in the Herbarium of the Department of Plant Pathology, Agricultural College, Guizhou University (HGUP). The ex-type cultures have been deposited in the Departmental Culture Collection (GUCC).

DNA extraction and sequencing

Upon reaching the border of a 90 mm diameter Petri dish, a sterile scalpel was used to transfer mycelium into a 1.5 mL centrifuge tube for the extraction of genomic DNA. This extraction was performed using PrepMan Ultra Reagent (Applied Biosystems, CA, USA) in line with the manufacturer's guidelines. The polymerase chain reaction (PCR) amplification was undertaken with a reaction volume of 25 µL. Primer pairs ITS5/ITS4 (White et al. 1990), LR0R/LR5 (Vilgalys and Hester 1990), dRPB2-5F/dRPB2-7cR (Voglmayr et al. 2016), NS1/NS4 (White et al. 1990), Bt2a/Bt2b (Glass and Donaldson 1995), EF1-728F/EF-2 (O'Donnell et al. 1998, Carbone and Kohn 1999) were used to amplify the internal transcribed spacer regions (ITS), partial large subunit nrRNA (LSU) gene, partial DNA-directed RNA polymerase II second largest subunit (*rpb2*), 18S small subunit ribosomal RNA (SSU), partial beta-tubulin (*tub2*) gene, and translation elongation factor 1-alpha (*tef1-α*) gene sequence fragments, respectively.

The PCR thermal cycle program used for amplifying of ITS, LSU, *rpb2*, SSU, *tub2*, and *tef1-α* started with an initial denaturation at 95 °C for 5 minutes. This was followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 54 °C for 30 s, elongation at 72 °C for one minute each, and a final extension step at 72 °C lasting 10 minutes. Sangon Biotech (Chengdu, China) handled the purification and sequencing of PCR amplicons. Sequences meeting the quality criteria were submitted to GenBank, and their corresponding accession numbers are listed in Table 1, which also contains a complete list of all the strains utilized in this research.

Phylogenetic analyses

Reference sequences obtained from GenBank (Table 1) were utilized to assist with the phylogenetic analyses. Multiple sequence alignments were created using the online platform of MAFFT v.7.307 (http://mafft.cbrc.jp/alignment/server/) (Katoh and Standley 2016). AliView (Larsson 2014) was utilized for manual refinement, with terminal ends and ambiguous regions of the alignment being manually excised. Phylogenetic analyses were performed using concatenated sequences from the six (ITS, LSU, *rpb2*, SSU, *tub2*, and *tef1-α*) through Maximum Likelihood (ML), and Bayesian Inference (BI) methodologies.

Table 1. Species and GenBank accession numbers of DNA sequences used in in the phylogenetic analysis.

Species name	Voucher specimens	GenBank Accession numbers					
		ITS	LSU	*rpb2*	SSU	*tef1-α*	*tub2*
Byssothecium circinans	CBS675.92	OM337536	GU205217	DQ767646	GU205235	–	–
Haplohelminthosporium calami	MFLUCC18-0074*	MT928158	MT928156	–	MT928160	–	–
Helminthosporiella astilbacea	COAD2126	MG668862	–	–	–	–	–
Helminthosporiella stilbacea	MFLUCC15-0813*	MT928159	MT928157	–	MT928161	–	–
Helminthosporiella stilbacea	CPHmZC-01	KX228298	KX228355	–	–	–	–
Helminthosporium maquaticum	MFLUCC15-0357 = S-096*	KU697302	KU697306	–	KU697310	–	–
Helminthosporium austriacum	CBS139924 = L132*	KY984301	KY984301	KY984365	KY984420	–	–
Helminthosporium austriacum	CBS14238 = L169	KY984303	KY984303	KY984367	–	–	–
Helminthosporium austriacum	L137	KY984302	KY984302	KY984366	–	–	–
Helminthosporium caespitosum	CBS484.77 = L99*	JQ044429	JQ044448	KY984370	KY984421	–	–
Helminthosporium caespitosum	L141	KY984305	KY984305	KY984368	–	–	–
Helminthosporium caespitosum	L151	KY984306	KY984306	KY984369	–	–	–
Helminthosporium chengduense	UESTC22.0024 = YQ071048 = CGMCC	ON557751	ON557745	ON563073	ON557757	–	–
Helminthosporium chengduense	UESTC22.0025 = YQ071047	ON557750	ON557744	ON563072	ON557756	–	–
Helminthosporium chiangraiense	MFLUCC21-0087*	MZ538504	MZ538538	–	–	–	–
Helminthosporium chlorophorae	BRIP14521	AF120259	–	–	–	–	–
Helminthosporium dalbergiae	MAFF243853 = H4628 = TS36	LC014555	AB807521	–	AB797231	–	–
Helminthosporium endiandrae	CBS138902 = CPC22194*	KP004450	KP004478	–	–	–	–
Helminthosporium erythrinicola	CPC35291 = CBS145569*	NR_165563	MK876432	MK876486	–	–	–
Helminthosporium genistae	CBS142597 = L142*	KY984310	KY984310	KY984374	–	–	–
Helminthosporium genistae	CBS139922 = L129	KY984309	KY984309	KY984373	KY984423	–	–
Helminthosporium genistae	CBS139921 = L128	KY984308	KY984308	KY984372	KY984422	–	–
Helminthosporium guizhouense	**GUCC24-0011***	**PP915799**	**PP949847**	**PP947940**	**PP949912**	–	–
Helminthosporium guizhouense	**GUCC24-0012**	**PP915800**	**PP949848**	**PP947941**	**PP949913**	–	–
Helminthosporium guizhouense	**GUCC24-0013**	**PP915801**	**PP949849**	**PP947942**	**PP949914**	–	–
Helminthosporium hispanicum	CBS136917 = L109*	KY984318	KY984318	KY984381	KY984424	–	–
Helminthosporium juglandinum	CBS136922 = L118*	KY984321	KY984321	KY984384	–	–	–
Helminthosporium juglandinum	CBS136911 = L97	KY984322	KY984322	KY984385	KY984425	–	–
Helminthosporium juglandinum	CBS136912 = L101	KY984319	KY984319	KY984382	–	–	–
Helminthosporium juglandinum	CBS136913 = L102	KY984320	KY984320	KY984383	–	–	–
Helminthosporium leucadendri	CBS135133 = CPC19345*	KF251150	KF251654	KF252159	–	–	–
Helminthosporium livistonae	CPC32158 = CBS144413*	NR_160348	NG_064539	–	–	–	–

Species name	Voucher specimens	GenBank Accession numbers					
		ITS	LSU	*rpb2*	SSU	*tef1-α*	*tub2*
Helminthosporium magnisporum	MAFF239278 = H4627 = TS33*	AB811452	AB807522	–	AB797232	–	–
Helminthosporium massarinum	CBS139690 = JCM13095 = MAFF239605 = KT1564*	AB809629	AB807524	–	AB797234	–	–
Helminthosporium massarinum	JCM13094 = MAFF239604 = KT838*	AB809628	AB807523	–	AB797233	–	–
Helminthosporium microsorum	CBS136910 = L96*	KY984329	KY984329	KY984390	KY984427	–	–
Helminthosporium microsorum	L94	KY984327	KY984327	KY984388	KY984426	–	–
Helminthosporium microsorum	CBS136916 = L108	KY984323	KY984323	KY984386	–	–	–
Helminthosporium microsorum	L95	KY984328	KY984328	KY984389	–	–	–
Helminthosporium nanjingensis	HHAUF020380 = ZM020380	KF192322	–	–	–	–	–
Helminthosporium oligosporum	CBS136909 = L93*	KY984333	KY984333	KY984394	–	–	–
Helminthosporium oligosporum	CBS136908 = L92	KY984332	KY984332	KY984393	KY984428	–	–
Helminthosporium oligosporum	L106	KY984330	KY984330	KY984391	–	–	–
Helminthosporium quercinum	CBS136921 = L90*	KY984339	KY984339	KY984400	KY984429	–	–
Helminthosporium quercinum	CBS112393	KY984334	KY984334	KY984395	–	–	–
Helminthosporium quercinum	CBS136915 = L107	KY984336	KY984336	KY984397	–	–	–
Helminthosporium solani	CBS365.75	KY984341	KY984341	KY984402	KY984430	–	–
Helminthosporium solani	CBS640.85	KY984342	KY984342	KY984403	–	–	–
Helminthosporium submersum	MFLUCC16-1360*	–	MG098787	–	MG098796	–	–
Helminthosporium submersum	MFLUCC16-1290PT	MG098780	MG098788	MG098592	MG098797	–	–
Helminthosporium submersum	UESTCC22.0021 = Sara08_3 = CGMCC	ON557753	ON557747	ON563075	ON557759	–	–
Helminthosporium syzygii	CPC35312 = CBS145570*	NR_165564	MK876433	MK876487	–	–	–
Helminthosporium tiliae	CBS136907 = L88*	KY984345	KY984345	KY984406	KY984431	–	–
Helminthosporium tiliae	CBS136906 = L87	KY984344	KY984344	KY984405	–	–	–
Helminthosporium tiliae	L171	KY984343	KY984343	KY984404	–	–	–
Helminthosporium velutinum	CBS139923 = L131*	KY984352	KY984352	KY984413	KY984432	–	–
Helminthosporium velutinum	L98	KY984359	KY984359	KY984417	KY984433	–	–
Helminthosporium velutinum	CBS136924 = L115	KY984347	KY984347	KY984408	–	–	–
Helminthosporium velutinum	L116	KY984348	KY984348	KY984409	–	–	–
Helminthosporium velutinum	L117	KY984349	KY984349	KY984410	–	–	–
Helminthosporium velutinum	UESTCC22.0022 = BY14_2 = CGMCC3.23572	ON557755	ON557749	–	ON557761	–	–
Helminthosporium chinense	UESTCC22.0026 = YQ071,005 = CGMCC3.23570*	ON557754	ON557748	–	ON557760	–	–
Massarina cisti	CBS266.62 = JCM14140*	LC014568	AB807539	FJ795464	AB797249	–	–

Species name	Voucher specimens	GenBank Accession numbers					
		ITS	LSU	*rpb2*	SSU	*tef1-α*	*tub2*
Massarina eburnea	CBS473.64	OM337528	GU301840	GU371732	GU296170	–	–
Massarina eburnea	CBS139697 = JCM14422 = H3953	LC014569	AB521735	–	AB521718	–	–
Massarina pandanicola	MFLUCC17-0596 = KUMCC17-0293*	MG646958	MG646947	–	MG646979	–	–
Periconia pseudodigitata	KT1395 = HHUF29370 = CBS139699 = JCM13166 = MAFF239676*	NR_153490	NG_059396	–	NG_064850	–	–
Pseudodidymosphaeria spartii	MFLUCC13-0273	KP325434	KP325436	–	KP325438	–	–
Pseudodidymosphaeria spartii	MFLUCC14-1212	KP325435	KP325437	–	KP325439	–	–
Pseudosplanchnonema phorcioides	L16 = CBS122935	KY984360	KY984360	KY984418	KY984434	–	–
Pseudosplanchnonema phorcioides	MFLUCC13-0533 = CGMCC3.17583	–	KM875454	–	KM875455	–	–
Pseudosplanchnonema phorcioides	MFLUCC13-0611	KP683375	KP683376	–	KP683377	–	–
Pseudosplanchnonema phorcioides	MFLUCC14-0618	KP683372	KP683373	–	KP683374	–	–
Semifissispora natalis	CPC25383 = CBS140659*	KT950846	KT950858	–	–	–	–
Semifissispora rotundata	CBS172.93 = CPC549	KT950847	KT950859	–	–	–	–
Semifissispora tooloomensis	CBS143431 = CPC31680*	NR_156674	NG_058526	–	–	–	–
Stagonospora duoseptata	CBS135093 = S618*	KF251255	KF251758	KF252260	–	–	–
Stagonospora imperaticola	MFLUCC15-0026 = ICMP21563*	KY706143	KY706133	KY706149	KY706138	–	–
Stagonospora multiseptata	MFLUCC15-0449 = ICMP21562*	NR_165854	NG_068239	–	–	–	–
Stagonospora paludosa	CBS135088*	KF251257	KF251760	KF252262	–	–	–
Stagonospora perfecta	KT1726A = JCM13099 = MAFF239609	AB809642	AB807579	–	AB797289	–	–
Stagonospora perfecta	CBS135099 = S656*	KF251258	KF251761	KF252263	–	–	–
Stagonospora pseudocaricis	CBS135132 = S610*	KF251259	KF251763	KF252265	–	–	–
Stagonospora pseudopaludosa	CPC22654 = CBS136424*	NR_137840	NG_058052	–	–	–	–
Stagonospora pseudoperfecta	CBS120236 = JCM13097 = MAFF239607*	AB809641	AB807577	–	AB797287	–	–
Stagonospora tainanensis	KT1866 = MAFF243860	AB809643	AB807580	–	AB797290	–	–
Stagonospora trichophoricola	CBS136764 = D652*	NR_156586	NG_058081	KJ869232	–	–	–
Stagonospora uniseptata	CBS135090 = S611*	KF251264	KF251767	KF252269	–	–	–
Stagonospora uniseptata	S607 = CPC22151	KF251265	KF251768	KF252270	–	–	–
Stagonospora uniseptata	S608 = CPC22150	KF251266	KF251769	KF252271	–	–	–

Species name	Voucher specimens	GenBank Accession numbers					
		ITS	LSU	*rpb2*	SSU	*tef1-α*	*tub2*
Suttonomyces clematidis	MFLUCC14-0240 = GUCC18	–	KP842917	–	KP842920	–	–
Suttonomyces rosae	MFLUCC15-0051*	MG828973	MG829085	–	MG829185	–	–
Synhelminthosporium synnematoferum	UESTCC22.0023 = HLG072894 = CGMCC3.23574*	ON557752	ON557746	ON563074	ON557758	–	–
Apiospora malaysiana	CBS 102053	KF144896	–	–	–	KF145030	KF144988
Apiospora pseudoparenchymatica	LC7234*	KY494743	–	–	–	KY705139	KY705211
Nigrospora aurantiaca	CGMCC 3.18130*	KX986064	–	–	–	KY019295	KY019465
Nigrospora aurantiaca	LC7034	KX986093	–	–	–	KY019394	KY019598
Nigrospora bambusae	CGMCC 3.18327*	KY385307	–	–	–	KY385313	KY385319
Nigrospora bambusae	LC7245	KY385305	–	–	–	KY385315	KY385321
Nigrospora brasiliensis	CMM 1214*	KY569629	–	–	–	MK753271	MK720816
Nigrospora brasiliensis	CMM 1217	KY569630	–	–	–	MK753272	MK720817
Nigrospora camelliae-sinensis	CGMCC 3.18125*	KX985986	–	–	–	KY019293	KY019460
Nigrospora chinensis	LC6851	KX986049	–	–	–	KY019450	KY019579
Nigrospora chinensis	CGMCC 3.18127*	KX986023	–	–	–	KY019422	KY019462
Nigrospora covidalis	CGMCC 3.20538*	OK335209	–	–	–	OK431485	OK431479
Nigrospora covidalis	LC158337	OK335210	–	–	–	OK431486	OK431480
Nigrospora endophytica	URM8712 = A.R.M. 687	OM265226	–	–	–	OP572415	OP572418
Nigrospora endophytica	URM8462 = A.R.M. 973*	OM265233	–	–	–	OP572416	OP572420
Nigrospora falsivesicularis	CGMCC 3.19678*	MN215778	–	–	–	MN264017	MN329942
Nigrospora falsivesicularis	LC13553	MN215779	–	–	–	MN264018	MN329943
Nigrospora globospora	CGMCC 3.20539*	OK335211	–	–	–	OK431487	OK431481
Nigrospora globospora	LC15839	OK335212	–	–	–	OK431488	OK431482
Nigrospora gorlenkoana	CBS 480.73*	KX986048	–	–	–	KY019420	KY019456
Nigrospora guangdongensis	CFCC:53917*	MT017509	–	–	–	MT024493	MT024495
Nigrospora guilinensis	LC7301	KX986063	–	–	–	KY019404	KY019608
Nigrospora guilinensis	CGMCC 3.18124*	KX985983	–	–	–	KY019292	KY019459
Nigrospora hainanensis	CGMCC 3.18129*	KX986091	–	–	–	KY019415	KY019464
Nigrospora hainanensis	URM8714 = A.R.M.967	OM265228	–	–	–	OM642834	OM793057
Nigrospora hainanensis	URM8715 = A.R.M.968	OM265229	–	–	–	OM642835	OM793058
Nigrospora lacticolonia	CGMCC 3.18123*	KX985978	–	–	–	KY019291	KY019458
Nigrospora lacticolonia	URM8713 = A.R.M. 921	OM265227	–	–	–	OM642833	OM642838
Nigrospora magnoliae	MFLUCC 19–0112*	MW285092	–	–	–	–	MW438334
Nigrospora manihoticola	URM8461 = A.R.M. 645*	OM265224	–	–	–	OM914791	OM869479
Nigrospora musae	CBS 319.34*	KX986076	–	–	–	KY019419	KY019455

Species name	Voucher specimens	GenBank Accession numbers					
		ITS	LSU	*rpb2*	SSU	*tef1-α*	*tub2*
Nigrospora musae	LC6385	KX986042	–	–	–	KY019371	KY019567
Nigrospora oryzae	LC2724	KX985959	–	–	–	KY019312	KY019486
Nigrospora oryzae	LC4265	KX985994	–	–	–	KY019335	KY019518
Nigrospora osmanthi	CGMCC 3.18126*	KX986010	–	–	–	KY019421	KY019461
Nigrospora osmanthi	LC4487	KX986017	–	–	–	KY019438	KY019540
Nigrospora pernambucoensis	URM8711 = A.R.M.651	OM265225	–	–	–	OM914792	OM869480
Nigrospora pernambucoensis	URM8463 = A.R.M. 974*	OM265234	–	–	–	OM914793	OM869481
Nigrospora philosophiae-doctoris	CGMCC 3.20540*	OK335214	–	–	–	OK431490	OK431484
Nigrospora pyriformis	CGMCC 3.18122*	KX985940	–	–	–	KY019290	KY019457
Nigrospora pyriformis	URM8716 = A.R.M.970	OM265231	–	–	–	OM513904	OM642839
Nigrospora rubi	LC2698*	KX985948	–	–	–	KY019302	KY019475
*Nigrospora sacchari*cola	LC12057	MN215789	–	–	–	MN264028	MN329952
*Nigrospora sacchari*cola	CGMCC 3.19362*	MN215788	–	–	–	MN264027	MN329951
Nigrospora sacchari-ofcinarum	CGMCC 3.19335*	MN215791	–	–	–	MN264030	MN329954
Nigrospora sacchari-ofcinarum	LC13531	MN215792	–	–	–	MN264031	MN329955
Nigrospora singularis	CGMCC 3.19334*	MN215793	–	–	–	MN264032	MN329956
Nigrospora singularis	LC12068	MN215794	–	–	–	MN264033	MN329957
Nigrospora sphaerica	LC2839	KX985964	–	–	–	KY019317	KY019491
Nigrospora sphaerica	LC2840	KX985965	–	–	–	KY019318	KY019492
Nigrospora sp. 1	LC2725	KX985960	–	–	–	KY019313	KY019487
Nigrospora sp. 1	LC4566	KX986022	–	–	–	KY019354	KY019545
Nigrospora sp. 2	LC6704	KX986047	–	–	–	KY019373	KY019571
Nigrospora stoneae	BRIP 75022a	OR608744	–	–	–	OR604065	OR604067
Nigrospora vesicularis	LC0322	KX985939	–	–	–	KY019296	KY019467
Nigrospora vesicularis	CGMCC 3.18128*	KX986088	–	–	–	KY019294	KY019463
Nigrospora vesicularifera	CGMCC 3.19333*	MN215812	–	–	–	MN264051	MN329975
Nigrospora vesicularifera	URM8718 = A.R.M.975	OM265235	–	–	–	OM513905	OM642840
Nigrospora yunnanensis	**GUCC24-0008***	**PP915796**	–	–	–	**PP947933**	**PP947937**
Nigrospora yunnanensis	**GUCC24-0009**	**PP915797**	–	–	–	**PP947934**	**PP947938**
Nigrospora yunnanensis	**GUCC24-0010**	**PP915798**	–	–	–	**PP947935**	**PP947939**
Nigrospora zimmermanii	CBS 290.62*	KY385309	–	–	–	KY385311	KY385317
Nigrospora zimmermanii	CBS 984.69	KY385310	–	–	–	KY385316	KY385322

* = Type specimens. Our strains in this study were in bold.

The Maximum Likelihood analysis was executed on the IQ-TREE web server (http://iqtree.cibiv.univie.ac.at/) (Trifinopoulos et al. 2016). The models is: In Fig. 1, TIM2e+I+G4 for ITS, TIM2e+I+G4 for LSU, TN+F+I+G4 for *rpb2* and K2P+G4 for SSU; In Fig. 2, TNe+I+G4 for ITS, TN+F+I+G4 for *tef1-a*, HKY+F+I+G4

for *tub2*. The number of bootstrap alignments is 1000 (Nguyen et al. 2015). Bayesian analysis (BI) was carried out using PhyloSuite v.1.2.2 as a tool (Zhang et al. 2019). In Fig. 1, SYM+I+G4 as the optimal model for ITS and LSU, HKY+F+I+G4 as the best-fit model for *rpb2*, K2P G4 as the optimal model for SSU; In Fig. 2, SYM+I+G4 as the optimal model for ITS, HKY+F+I+G4 as the best-fit model for *tef1-α* and *tub2*. Four chains were run for 10,000,000 generations and sampled every 500 generations. The initial 25% of the resulting trees were discarded as burn-in, and the remaining trees were used for calculating posterior probabilities in the majority rule consensus tree. The final phylogenetic topology was visualized with FigTree v.1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/) and was modified in Microsoft Office PowerPoint 2019.

Results

Phylogenetic analyses

For the *Helminthosporium* and related genera (Fig. 1), the phylogenetic trees accommodated 96 sequences listed in Table 1. The strains GUCC24-0011, GUCC24-0012, and GUCC24-0013 were characterized based on their molecular properties, specifically sequencing of the ITS, LSU, *rpb2*, and SSU genes regions. An outgroup consisting of the type strain KT1395 of *Periconia pseudodigitata* was also included in the study based on concatenated datasets, as shown in Table 1. The combined alignment consists of 5596 characters, including ITS (1766 characters), LSU (1648 characters), *rpb2* (1114 characters), and SSU (1065 characters) regions. We constructed two phylogenetic trees: an ML tree and a BI tree. The ML tree was selected to represent the phylogenetic relationship of different *Helminthosporium* taxa (Fig. 1). *Helminthosporium guizhouense* (GUCC24-0011, GUCC24-0012, and GUCC24-0013) was found to be a sister taxon to *H. caespitosum* (CBS484.77, L141, and L151) with high support values from both ML and BI analyses (ML/BI: 100/1).

For the *Nigrospora* and related genera (Fig. 2), the phylogenetic trees accommodated 68 sequences listed in Table 1. The strains GUCC24-0008, GUCC24-0009, and GUCC24-0010 were characterized based on their molecular properties, and accurate sequencing of the ITS, *tef1-α*, and *tub2* gene regions. *Apiospora malaysiana* (CBS 102053) and *A. pseudoparenchymatica* (LC7234) were selected as outgroups. The combined alignment consists of 1523 characters, including ITS (571 characters), *tef1-α* (562 characters), *tub2* (385 characters) regions. We constructed two phylogenetic trees: an ML tree and a BI tree. The ML tree was selected to represent the phylogenetic relationship of different *Nigrospora* taxa (Fig. 2). *Nigrospora yunnanensis* (GUCC24-0011, GUCC24-0012, and GUCC24-0013) formed an independent branch without the DNA base differences in three loci supported by strong statistic data (ML/BI: 100/1) and were adjacent to the branch of *N. falsivesicularis* (CGMCC 3.19678 and LC13553), *N. vesicularis* (LC0322 and CGMCC 3.18128), *N. aurantiaca* (CGMCC 3.18130 and LC7034), *N. stoneae* (BRIP 75022a), *N. lacticolonia* (CGMCC 3.18123 and URM8713), *N. osmanthi* (CGMCC 3.18126 and LC4487), *N. endophytica* (URM8712 and URM8462), *N. pernambucoensis* (URM8711 and URM8463), and *N. guilinensis* (LC730 and CGMCC 3.18124) (ML/BI: 97/0.99).

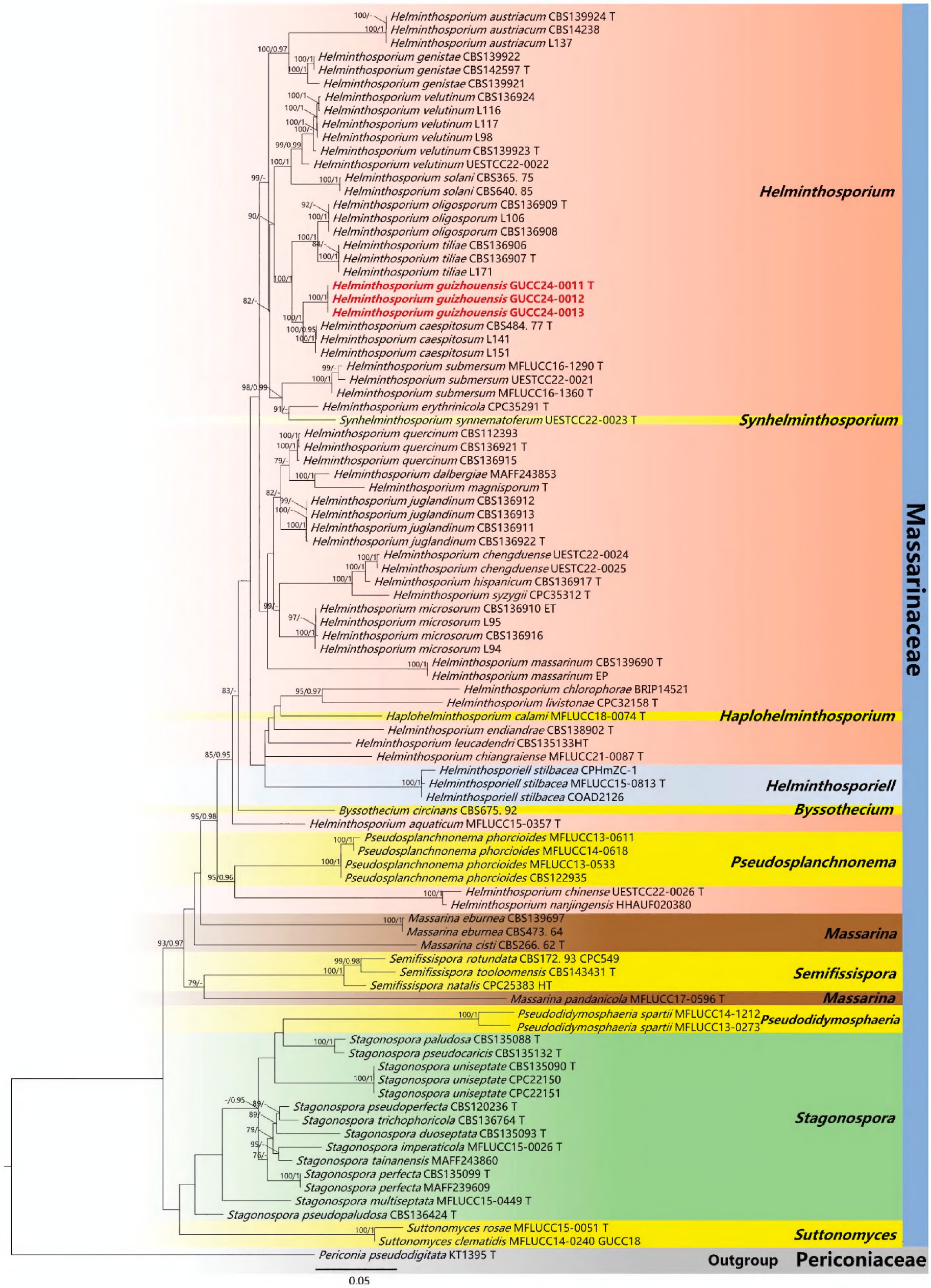

Figure 1. The maximum parsimony tree of 96 *Helminthosporium* taxa is based on ITS, LSU, *rpb2*, and SSU genes. The tree was rooted with *Periconia pseudodigitata* (KT1395). Bootstrap support values for ML greater than 75% and Bayesian posterior probabilities greater than 0.95 are given near nodes, respectively. The new isolates were in red. Ex-type strains were marked by T. The scale bar indicates 0.05 expected changes per site.

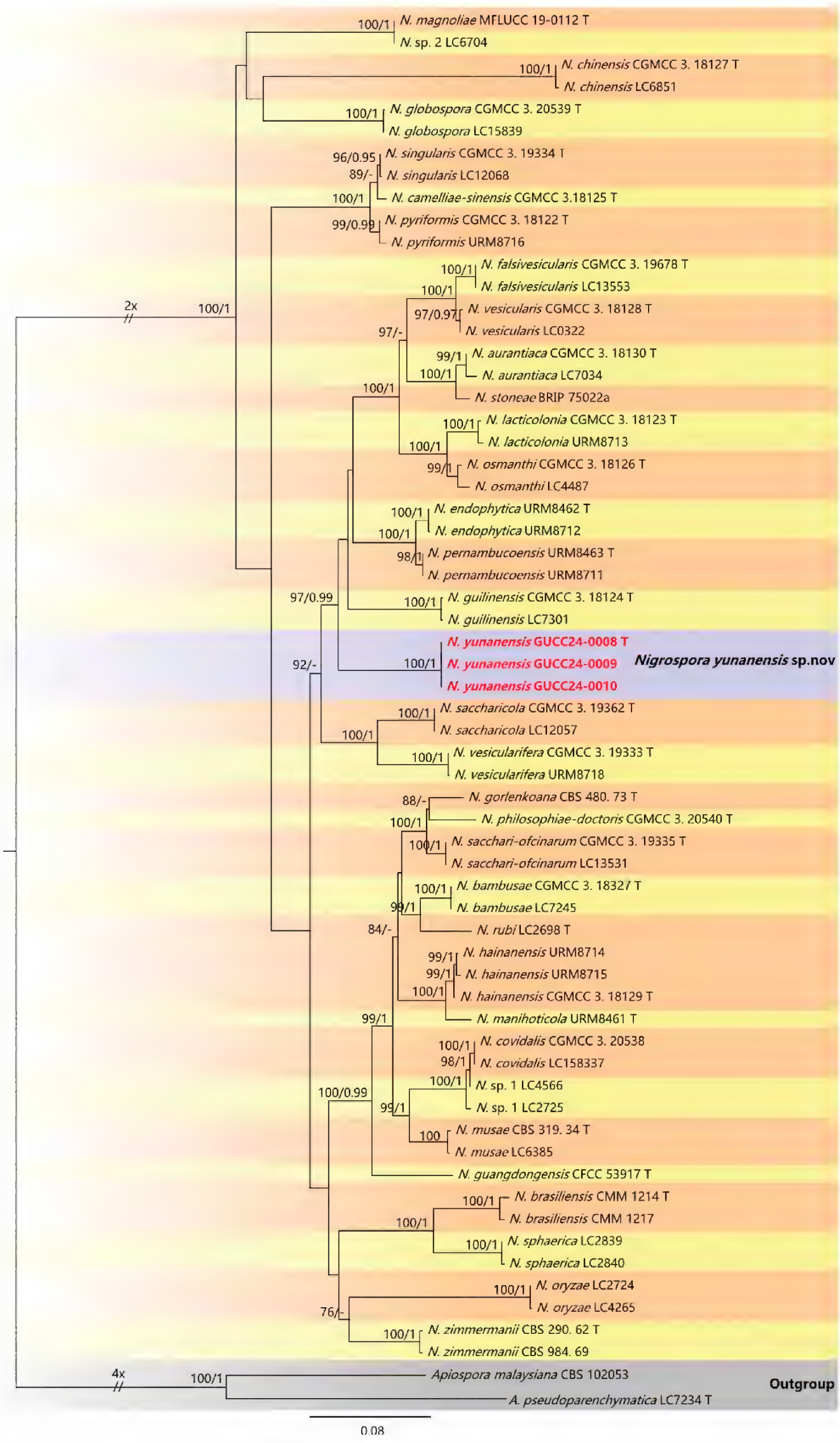
N. magnoliae MFLUCC 19-0112 T
N. sp. 2 LC6704
N. chinensis CGMCC 3. 18127 T
N. chinensis LC6851
N. globospora CGMCC 3. 20539 T
N. globospora LC15839
N. singularis CGMCC 3. 19334 T
N. singularis LC12068
N. camelliae-sinensis CGMCC 3.18125 T
N. pyriformis CGMCC 3. 18122 T
N. pyriformis URM8716
N. falsivesicularis CGMCC 3. 19678 T
N. falsivesicularis LC13553
N. vesicularis CGMCC 3. 18128 T
N. vesicularis LC0322
N. aurantiaca CGMCC 3. 18130 T
N. aurantiaca LC7034
N. stoneae BRIP 75022a
N. lacticolonia CGMCC 3. 18123 T
N. lacticolonia URM8713
N. osmanthi CGMCC 3. 18126 T
N. osmanthi LC4487
N. endophytica URM8462 T
N. endophytica URM8712
N. pernambucoensis URM8463 T
N. pernambucoensis URM8711
N. guilinensis CGMCC 3. 18124 T
N. guilinensis LC7301
N. yunanensis GUCC24-0008 T
N. yunanensis GUCC24-0009
N. yunanensis GUCC24-0010
Nigrospora yunanensis sp.nov
N. saccharicola CGMCC 3. 19362 T
N. saccharicola LC12057
N. vesicularifera CGMCC 3. 19333 T
N. vesicularifera URM8718
N. gorlenkoana CBS 480. 73 T
N. philosophiae-doctoris CGMCC 3. 20540 T
N. sacchari-ofcinarum CGMCC 3. 19335 T
N. sacchari-ofcinarum LC13531
N. bambusae CGMCC 3. 18321 T
N. bambusae LC7245
N. rubi LC2698 T
N. hainanensis URM8714
N. hainanensis URM8715
N. hainanensis CGMCC 3. 18129 T
N. manihoticola URM8461 T
N. covidalis CGMCC 3. 20538
N. covidalis LC158337
N. sp. 1 LC4566
N. sp. 1 LC2725
N. musae CBS 319. 34 T
N. musae LC6385
N. guangdongensis CFCC 53917 T
N. brasiliensis CMM 1214 T
N. brasiliensis CMM 1217
N. sphaerica LC2839
N. sphaerica LC2840
N. oryzae LC2724
N. oryzae LC4265
N. zimmermanii CBS 290. 62 T
N. zimmermanii CBS 984. 69
Apiospora malaysiana CBS 102053
A. pseudoparenchymatica LC7234 T
Outgroup
2x
4x
0.08

Figure 2. The maximum parsimony tree of 68 *Nigrospora* taxa is based on ITS, *tef1-α*, and *tub2* genes. The tree was rooted with *Apiospora malaysiana* (CBS 102053) and *A. pseudoparenchymatica* (LC7234). Bootstrap support values for ML greater than 75% and Bayesian posterior probabilities greater than 0.95 are given near nodes, respectively. The new isolates were in red. Ex-type strains were marked by T. The scale bar indicates 0.08 expected changes per site.

Taxonomy

***Helminthosporium guizhouense* M.T. Zou & Yong Wang bis, sp. nov.**
MycoBank No: 854537
Fig. 3a–r

Etymology. The name refers to Guizhou, the province where the fungus was collected.

Diagnosis. *Helminthosporium guizhouense* can easily be distinguished from *H. caespitosum* by its narrower conidia (13–16 µm vs. 27.3–35.5 µm).

Type. China • Guizhou Province, QianXi City; 26°56'11.58"N, 105°55'15.46"E; 1235 m; 24 January 2023; from rotten dead branch of *Juglans regia*, coll. M.T. Zou; HGUP24-0007 (holotype); ex-type culture GUCC23-0011 (ITS: PP915799, LSU: PP949847, *rpb2*: PP947940; SSU: PP949912).

Description on the natural substrate. Colonies hairy, brown, or blackish-brown, in groups. Mycelium partly immersed in the substratum, towards the surface forming stroma-like aggregations of light to brown pseudoparenchymatous cells.

Culture characteristics. Colony on PDA 25 mm diam after 2 weeks in an incubator under dark conditions at 28 °C, irregular circular, fat, raised, undulate, rough, with white and denser mycelium at the center, with white to deep-gray to creamy yellow, entire margin; reverse cream to yellow, with dark yellowish-brown spots. Teleomorph: Unknown. Anamorph: Conidiophores macronematous, erect, straight, or slightly curved, cylindrical, smooth, 171–718 µm long, 12–25 µm wide at the base, tapering to 7–13.5 µm near the apex, arising solitary or in fascicles from the stroma cells, erect, simple, straight or flexuous, thick-walled, brown to dark brown, with sympodial proliferation, 1–13-septate. Conidia 61–114 × 13–16 µm ($\bar{x}$ = 85 × 18, n = 45), gradually tapering to 3–7 µm ($\bar{x}$ = 5, n = 45) at the distal end, with a 4–10 µm ($\bar{x}$ = 6, n = 42) wide, blackish-brown to black scars at the base, straight or flexuous, solitary, obclavate to rostrate, smooth-walled, hyaline, pale golden brown to brown, 8–12-distoseptate, with angular lumina; wall up to 6 µm thick.

Habit. Saprobic on decaying wood of *Juglans regia*.

Distribution. China, Guizhou Province, Qianxi City

Other material examined. China • Guizhou Province, Qianxi City; 105°92'E, 26°93'N; 1235 m; 24 January 2023; from rotten dead branch of *Juglans regia*, coll. M.T. Zou, HGUP24-0008 (holotype); living culture GUCC24-0011, GUCC24-0012, and GUCC24-0013.

Notes. Based on the multi-gene phylogenetic tree (Fig. 1), our strains are clustered in a distinct branch adjacent to the strain of *Helminthosporium caespitosum* (CBS 484.77). Topologically, there is a clear genetic distance between these taxa with ML-BS = 100%, BYPP = 1 support. When comparing the ITS, LSU, *rpb2*, and SSU nucleotides of *H. guizhouense* with *H. caespitosum* in the clade, there are 22 bp (0 gap) differences of 569 bp in ITS, 2 bp (0 gap) differences of 904 bp in LSU, and 38 bp (0 gap) differences of 401 bp in rpb2, and 4 bp (0 gap) differences of 1098 bp in SSU. Our collection of *H. guizhouense* (HGUP24-0008) differs significantly from the holotype of *H. caespitosum* (WU 38825 and WU 38826) (Voglmayr and Jaklitsch 2017) in the length of conidiophores (171–718 × 9.5–23 µm vs. 27–37 × 12.2–14.5 µm), the size of conidia (61–114 × 13–16 µm vs. 82–109 × 27.3–35.5 µm), the number of septa (8–12

Figure 3. *Helminthosporium guizhouense* sp. nov. (HGUP24-0008, holotype) on rotten dead branch of *Juglans regia* **a–c** colonies on the natural substrat; **d, e** culture on PDA after 2 weeks (**d** above **e** reverse) **f** conidiophore bases, stroma cells, and conidia **l** conidiophore **m** colony, conidiophores, and stroma cells **n** conidiophore **g–k, o–r** conidia. Scale bars: 1000 µm (**a**); 500 µm (**b, c**); 50µm (**f–r**).

vs. 6–10) and the wall thickness of angular lumina (6 µm vs. 8 µm). In addition, the colonies on the natural substrate of *H. guizhouense* are hairy, brown, or blackish brown, in groups, whereas *H. caespitosum* is dark-red-brown, scattered, or crowded. Through our analysis and classification process, we have identified these three strains as a new species *Helminthosporium guizhouense*.

***Nigrospora yunnanensis* M.T. Zou & Yong Wang bis, sp. nov.**

MycoBank No: 854538

Fig. 4a–k

Etymology. The name refers to Yunnan, the province where the fungus was collected.

Diagnosis. *Nigrospora yunnanensis* is characterized by black, globose conidia (16.2 × 14.4 µm).

Type. China • Yunnan Province: Lincang City; 23°40'26.08"N, 99°56'47.70"E; 1900 m; 22 Dec 2023; on *Juglans regia*, coll. M.T. Zou; HGUP24-0007 (holotype); ex-type culture GUCC24-0008 (ITS: PP915796, *tef1-α*: PP947933, *tub2*: PP947937).

Culture characteristics. Colonies on PDA reaching 90 mm diam after ten days at 25 °C. The anterior surface and posterior surface are white, while the mycelium is thick and fluffy. Colonies on OA reach 90 mm diam. after ten days at 25 °C. The mycelium is circular, filiform, fluffy, while the surface and reverse are initially white, becoming gray to black, or black and producing a few black areas with age. Colonies on MEA reaching a diameter of 90 mm after ten days at 25 °C. The mycelium circular, filiform, thick, and fluffy. The surface and reverse are initially white, but they become gray to dark black with abundant black areas spreading from the periphery to the center as they age. Sexual morph undetermined. Asexual morph on OA: Hyphae 2.5–8 µm diam, smooth, hyaline to pale brown, branched, septate. Conidiophores smooth, hyaline to brown, branched, septate, sometimes reduced to conidiogenous cells. Conidiogenous cells (n = 30) 8–14 × 6–10 µm (av. = 10.4 × 8.2 µm), aggregated in clusters on hyphae, pale brown, subglobose to ampulliform. Conidia (n = 40) 14.5–18.5 × 11–17.5 µm (av. = 16.2 × 14.4 µm) solitary, globose to subglobose, black, shiny, smooth, aseptate.

Habitat. On *Juglans regia*.

Known distribution. China, Yunnan Province, Lincang city.

Additional material examined. China • Yunnan Province: Lincang city; 23°67'N, 99°94'E; 1900 m; 22 Dec 2023; on *Juglans regia*; coll. M.T. Zou; HGUP24-0007; living culture GUCC24-0008, GUCC24-0009, and GUCC24-0010.

Notes. Three isolates from walnut leaves were obtained in this study and clustered in a well-supported clade distinguished from other known species (Fig. 4). *Nigrospora yunnanensis* formed an independent branch. Morphological differences (Table 2) support that they belong to different taxa.

Discussion

In the family Massarinaceae, along with *Helminthosporium*, there are ten other accepted genera: *Byssothecium*, *Haplohelminthosporium*, *Helminthosporiella*, *Massarina*, *Mirohelminthosporium*, *Pseudodidymosphaeria*, *Pseudosplanchnonema*, *Semifissispora*, *Stagonospora*, and *Suttonomyces* (Wijayawardene et al. 2022).

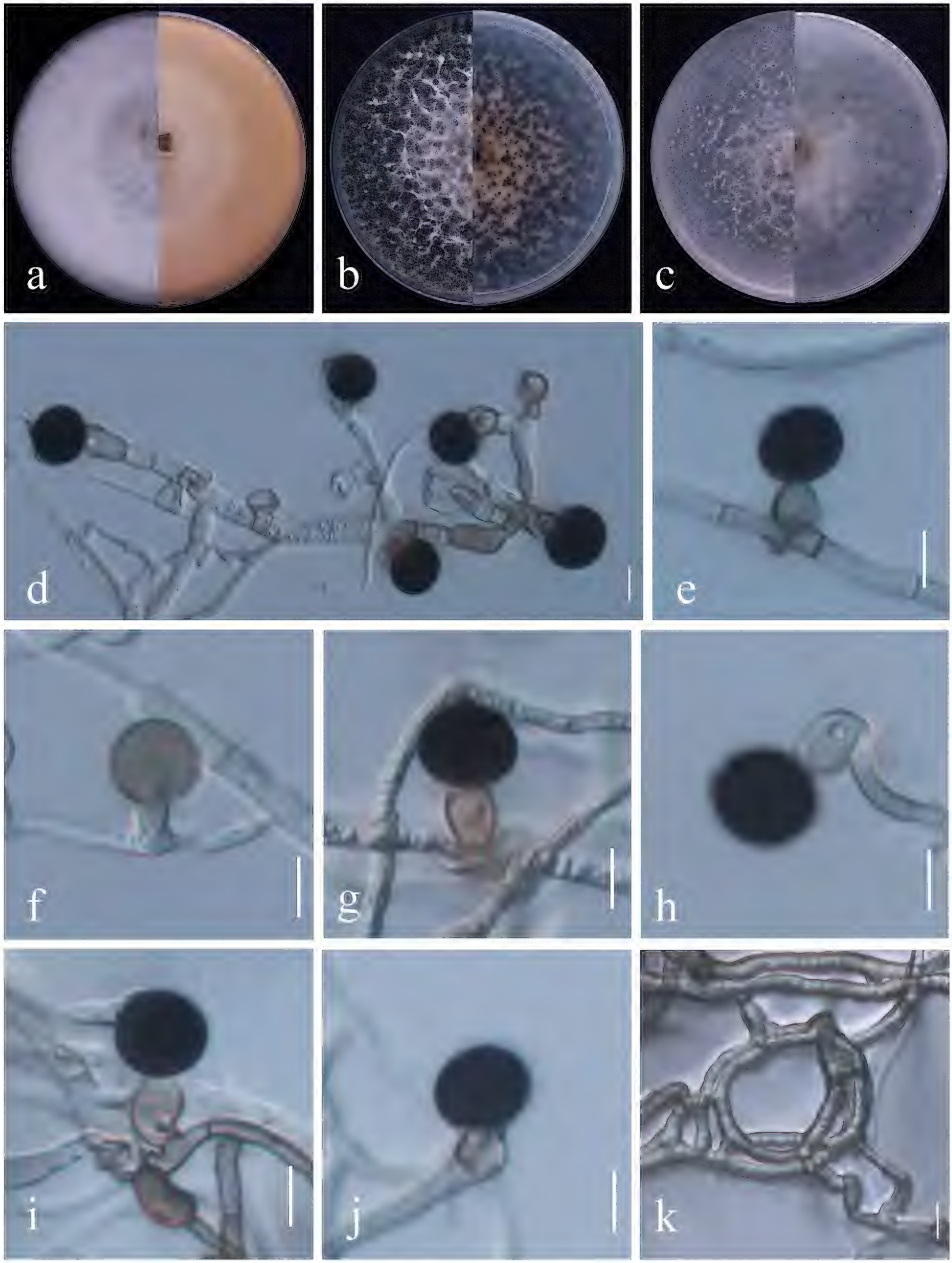

Figure 4. *Nigrospora yunnanensis* (GUCC23-0008) **a–c** culture characteristics on media after ten days (**a** on PDA **b** on CMA **c** on OA) **d–j** conidia attached to conidiogenous cells **k** coiled hyphae. Scale bars: 10 μm (**d–k**).

Table 2. Comparison of conidia and conidiogenous cells of *Nigrospora* species related to this study.

Species	Strain	Conidia (μm)	Conidiogenous cells (μm)	Reference
Nigrospora endophytica	URM8462	10–17.5	6.2–10	(Brito et al. 2023)
N. guilinensis	CGMCC 3.18124	11.5–15	6–11 × 4–7.5	(Wang et al. 2017)
N. pernambucoensis	URM8463	12.5–20	5–22.5 × 5–12.5	(Brito et al. 2023)
N. saccharicola	CGMCC 3.19362	13.5–16.5	7.5–10.5 × 5–7.5	(Raza et al. 2019)
N. vesicularifera	CGMCC 3.19333	11–19	7.5–10 × 12.5–15.5	(Raza et al. 2019)
N. yunnanensis	GUCC24-0008	14.4 × 16.2	6–10 × 8–14	This study

Helminthosporium is polyphyletic, as confirmed by Konta et al. (2021), and its members were found mixed with other taxa of *Byssothecium*, *Helminthosporiella*, and *Pseudosplanchnonema*. According to a study by Chen et al. (2022), a new genus (*Synhelminthosporium*) was identified through morphological examination and multi-locus phylogenetic analyses. Most *Helminthosporium* species are saprobic, primarily found on woody plant materials. However, some are plant pathogens, and others thrive on fungi, particularly in Diaporthales, but the role of these *Helminthosporium* species on their fungal hosts is still uncertain (Voglmayr and Jaklitsch 2017).

Nigrospora belongs to the Apiosporaceae, and are endophytes, saprobes, and plant pathogens, causing harm to economically important plant species within both forestry and agricultural domains. Examples include *N. oryzae* causing panicle branch rot disease on *Oryza sativa* in China (Liu et al. 2021), *N. sphaerica* causing Leaf blight disease of Cacao in the Philippines (Villanueva et al. 2023), *N. chinensis* causing stem spot on dragon fruit in China (Guo et al. 2024). *Nigrospora* is also a human pathogen. *N. oryzae* and *N. sphaerica* can cause human corneal keratitis (Ananya et al. 2014; Takayama et al. 2024). In addition, *N. yunnanensis* was isolated from *Juglans regia*, which could potentially be a pathogen for the walnuts.

The nutritional benefits of walnut kernels are substantial, as they are rich in fat, protein, vitamins, and minerals, while also containing essential compounds such as flavonoids and phenolic acids (Caglarirmak 2003). Moreover, walnuts are hosts to multiple forms of microfungi, including pathogens, endophytes, and saprobes (Zhang et al. 2024). Given this, it is crucial to undertake a comprehensive study of the microfungi present on walnuts in previously unexplored regions, for instance, the provinces of Yunnan and Guizhou in China, and to perform a thorough taxonomic classification of these microorganisms.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

Data curation: MZ. Formal analysis: FAO, KDH. Funding acquisition: XJP, YW. Supervision: XJP, YW. Writing – original draft: MZ. Writing – review and editing: FAO, XJP, YW, KDH.

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Data availability

All of the data that support the findings of this study are available in the main text.

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